7

PCT/AU 94 / 00694

Patent Office
Canberra

PRIORITY DOCUMENT

I, DAVID DANIEL CLARKE, ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 19 November 1993 in connection with Application No. PM 2540 for a patent by THE UNIVERSITY OF SYDNEY filed on 19 November 1993.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Twenty-fourth day of November 1994.

18

DAVID DANIEL CLARKE
DELEGATE OF COMMISSIONER OF PATENTS

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: *A method of preventing or controlling cataract*

The invention is described in the following statement:

GH&CO REF: P04828-WO:AMP

A METHOD FOR PREVENTING OR CONTROLLING CATARACT

Technical Field

The present invention relates to a method for preventing or controlling pathological changes which occur in association with cataract formation in the mammalian eye by reducing the amount of or inhibiting the action of transforming growth factor-beta(TGFß). The invention also relates to the use of inhibitors of TGFß to prevent or minimise "aftercataract"

Background Art

Cataract is an opacity of the lens that interferes with vision. It is one of the most common of eye diseases and, though it may occur at any time in life, it often accompanies aging. In the USA, for example, up to 45% of people aged between 74 and 89 years suffer from cataract. Currently, the most commonly used treatment for cataract is surgical removal of the lens cells and subsequent implantation of a synthetic replacement lens within the remaining lens capsule. However, implantation of a synthetic lens may only temporarily restore vision because residual cells associated with the lens capsule often grow rapidly and form new opacities. The latter condition is known as "aftercataract" or post-operative capsular opacification.

The TGFß family consists of a group of related proteins, the most extensively studied members being TGFß1, TGFß2 and TGFß3 and it has been reported that these are all present in the eye.

Disclosure of the Invention

In one aspect, the present invention provides a method of preventing or controlling cataract or cataract-like disorders in the eye of a mammalian subject which comprises administering to the subject an effective amount of one or more inhibitors of TGFß.

Preferably, the mammalian subject is a human being

but the present invention is also suitable for treating cataract or cataract-like disorders in other animals such as horses, cats, dogs or the like.

The biological activity of TGFß can be inhibited in a number of ways. One method of inhibiting the biological activity is by using an antibody directed against an active region of the TGFß molecule. TGFß biological activity can also be inhibited by the use of other molecules which sequester, inhibit or inactivate TGFß. For example, proteoglycans such as decorin can act as specific TGFß-binding proteins.

In another aspect, the present invention provides an ophthalmological formulation comprising one or more inhibitors of TGFß in a pharmaceutically acceptable carrier.

In a further aspect, the present invention provides a method of preventing or controlling "aftercataract" formation in the eye of a mammalian subject following lens implant surgery which comprises implanting in the eye of the subject a lens coated with one or more TGFß inhibitors.

In yet another aspect, the present invention provides a lens implant coated with one or more TGFß inhibitors.

In yet a further aspect, the present invention provides the use of inhibitors of TGFß in the manufacture of an ophthalmological formulation for preventing or controlling cataract or cataract-like disorders.

Modes of Carrying Out the Invention

The TGFß inhibitors according to the present invention can be administered either by topical application, by introduction into one or more chambers of the eye (for example, the anterior chamber), or as an intravenous injection at a site from which the inhibitors can be readily transported to the eye via the circulatory system. The treatment can be used as an adjunct to eye surgery to inhibit cataract-related changes that may occur as a result of surgical intervention as, for

example, in the formation of "aftercataract" following implantation of synthetic lens material. The present invention may also be suitable for treatment of individuals otherwise at greater than normal risk of cataract formation or of being exposed to elevated TGFß levels near the lens.

Ophthalmological formulations of the present invention are prepared according to conventional pharmaceutical formulating techniques. The carrier may be of any form depending on the form of preparation desired for administration and the formulation may optionally contain other therapeutic ingredients. Typically, one or more inhibitors of TGFß can be included in conventional irrigation solutions or viscoelastic solutions. Lens implants coated with one or more TGFß inhibitors may contain other therapeutic agents and may be prepared according to conventional techniques.

EXAMPLE 1.

Influence of TGFß alone and in combination with FGF on lens epithelial explants.

METHODS

Lens explants were prepared from both postnatal and adult rats and changes during 5 days culture with growth factor(s) were monitored by light and electron microscopy, immunolocalisation of laminin, heparan sulphate proteoglycan and fibre-specific crystallins, and crystallin ELISAs.

Each experiment involved culturing explants for up to 5 days without added growth factors (controls), with TGFß, with a combination of TGFß and FGF (TGFß/FGF), or with FGF alone. FGF is another growth factor that influences lens cell behaviour (Chamberlain and McAvoy, 1989; McAvoy et al., 1991). In some experiments, explants were prepared by a standard method used in our laboratory in which the adhering capsule serves as the substratum for the cells. In others, explants were inverted onto a laminin substratum. The latter method allows cell attachment, spreading and migration to be

monitored as well as providing good visualisation of individual cells.

Bovine brain basic FGF was prepared and stored at -20°C as described by Chamberlain and McAvoy (1989). Ultrapure natural human TGFß1 was obtained from Genzyme (Cambridge, MA) and stored at -80°C. Working stock solutions of TGFß and FGF were prepared (in culture medium or 1% bovine serum albumin-0.5 M NaCl in phosphate-buffered saline, respectively) and centrifuged at 10,000 g for 10 min at 4°C just before use.

Preparation and Culture of Lens Epithelial Explants: Standard Method

Eyes were removed from 10-day-old and 14-week-old Wistar rats under sterile conditions and placed in medium, that is, medium 199 containing bovine serum albumin and antibiotics as described by Hales et al (1992), pre-incubated at 37°C in 5% CO_2/air. Lenses were removed and incubated in 2 ml medium for 45-90 min (postnatal) or 1-2 hr (adult). Epithelia were then peeled away from fibres and pinned out with the cellular surface uppermost in culture dishes containing 2 ml medium as described by McAvoy and Fernon (1984). The whole epithelium was used, unless otherwise specified, and each dish contained 2-3 explants.

Approximately 3 hr after preparation of explants, medium was replaced (1 ml/dish) and 10 µl samples of stock solutions of TGFß and/or FGF were added, as required, to give final concentrations of 20 and 40 ng/ml, respectively. Explants were cultured for 5 days with daily monitoring by phase contrast microscopy. At appropriate times explants were processed for light or electron microcopy as described below. Alternatively, to assess the accumulation of fibre-specific crystallins, at the end of the culture period, explants were placed in 10 mM EDTA-0.02% Triton X-100, pH 10 (two explants in 200 µl) and stored at -20°C, then used for ß- and γ-crystallin ELISAs with standards ranging from 0-20 ng/well.

Preparation and Culture of Lens Epithelial Explants: on Laminin Substratum

This method is as described by Hales et al (1992). Briefly, on the day before the experiment, culture dishes were pre-coated with laminin. Whole explants were then prepared as described above, but with the cellular surface placed face down on the laminin and using lenses from 21-day-old rats; explants from rats of this age show a strong migratory response to FGF (unpublished observation). Each dish contained three explants. Growth factor treatments and culture conditions were as described for standard explants, except that a lower concentration of FGF, 2 ng/ml, was used to ensure that the main response to FGF alone was cellular migration rather than fibre differentiation. Responses were monitored daily by phase contrast microscopy.

Microscopy

Explants used for immunofluorescent localisation were collected at the end of the culture period, fixed in Carnoy's fixative for 20 min at room temperature, transferred to 70% ethanol, then covered with a drop of melted 2.5% agar, before dehydrating in ethanol and embedding in paraffin. Sections were cut perpendicular to the explant surface and stained with haematoxylin-phloxine or used for immunolocalisation of laminin, heparan sulphate proteoglycan (HSPG) or ß- and γ-crystallins. For each antibody and each explant 20-30 sections cut through the central region were examined, and at least two explants were processed for each growth factor treatment. Controls for non-specific fluorescence were included routinely, that is, sections were treated with non-immune rabbit serum instead of specific antibody. For whole mounts, explants were fixed in the culture dish with 100% ethanol and stained with haematoxylin-phloxine.

For ultrastructural studies, explants from 10-day-old rats were processed for transmission electron microscopy (TEM) and for scanning electron microscopy

(SEM) as described by Lovicu and McAvoy (1992); explants were collected at 3 or 5 days of culture. Explants from adult rats were processed for SEM only at 5 days. For both SEM and TEM, at least two explants were viewed for each treatment and, for TEM, 20-30 grids were viewed per explant.

RESULTS

Epithelial explants from postnatal rats (10 and 21 days old) were used for initial detailed studies. Because of the unusual nature of the observed responses to TGFß, a brief comparative study was then carried out using explants from adult rats.

Lens Explants from 10-day-old Rats: Standard Method

Phase contrast microscopy and SEM. In control and TGFß-treated explants the cells retained a characteristic epithelial cell morphology throughout the culture period, that is, they were present in a monolayer with cobblestone-like packing. In both cases, some cell debris was detected on the monolayer surface. In TGFß-treated explants only, single cells or small groups of cells were also occasionally detected on the monolayer surface. SEM of explants cultured for 5 days showed that the apical surface of some cells in TGFß-treated explants overlapped onto neighbouring cells.

TGFß/FGF- and FGF-treated explants were clearly distinguishable from controls within the first day of culture and indistinguishable from each other at this stage. Cells were irregularly packed and intercellular spaces were common, an explant morphology that is generally associated with active cell migration (McAvoy and Chamberlain, 1989; McAvoy, 1988). After 2 days culture some cells in the TGFß/FGF-treated, but not in the FGF-treated, explants were extensively elongated. The number of elongated cells varied between explants; they generally formed only a small proportion of the cellular population but because they often formed regular rows they were quite distinct from the other cells in the explant which appeared similar to those in the FGF-

treated explants. This marked difference between treatments was even more apparent at 3 days culture due to more cells becoming extensively elongated in TGFß/FGF-treated explants. At this stage SEM showed that many of the elongated cells were attached to neighbouring cells at multiple sites along their length.

By 4 and 5 days culture, most of the cells in TGFß/FGF-treated explants were in multilayers and all these explants had developed several regions where the cells were arranged in rosettes with elongated cells radiating out in a circular array from a focal point. Outside these rosettes, which occupied up to about 50% of the explant surface, there were some areas where similar extensively elongated cells were arranged in parallel arrays. Remaining cells were less elongated and appeared irregularly arrayed as in FGF-treated explants.

SEM showed that, in regions outside the rosettes and parallel arrays of extensively elongated cells, cells had numerous interlocking processes and appeared similar to the early differentiating fibres seen in explants treated with FGF alone. The morphological changes in explants from 10-day-old rats undergoing fibre differentiation in response to this concentration of FGF have been reported in detail elsewhere (Lovicu and McAvoy, 1992); multilayering and the formation of numerous interlocking processes are well-established features of this process (Lovicu and McAvoy, 1992; Lovicu and McAvoy, 1989). In the FGF/TGFß-treated explants, occasional patches of fibrillar extracellular matrix (ECM)-like material were noted on the explant surface. This matrix was dense and obscured the cells below.

TEM. Cells in explants cultured with FGF and TGFß/FGF for 5 days became multilayered and exhibited features of early fibre differentiation including elongation, sparse cytoplasmic organelles and nucleolar RNA particle aggregations; ball-and-socket joints typical of fibre differentiation were also detected. Additionally in TGFß/FGF-treated explants, cells exhibiting margination

of chromatin and cytoplasmic condensation were common, and membrane-bound cellular fragments and electron-dense bodies resembling secondary lysosomes were found within many cells that otherwise appeared normal. These features are characteristic of apoptosis or programmed cell death (Wyllie et al. 1980; Williams et al., 1992). Similar apoptotic changes were also detected in TGFß/FGF-treated explants at 3 days.

Pockets of ECM-like granular material were commonly detected between cells (and sometimes appeared to be within cells) in TGFß/FGF-treated explants. Often near the cell membrane this material was present in a laminar arrangement and coated pits and vesicles were common in such regions. Cells with prominent rough endoplasmic reticulum and Golgi, which also usually showed abundant arrays of microfilaments, were also found frequently in these explants.

In explants cultured with TGFß alone, the epithelial cells remained in a monolayer and were similar to controls except that, in the presence of TGFß, spaces were often present between cells. This, together with the overlapping of cells suggests that TGFß may be causing some disturbance of cell-cell interactions.

Immunohistochemical localisation of laminin and HSPG. The ECM molecules laminin and HSPG are both found in the normal lens capsule (Parmigiani and McAvoy, 1991; Mohan and Spiro, 1986) and, as expected, reactivity for both laminin and HSPG was detected in the capsule in all explants irrespective of treatment.

In TGFß/FGF-treated explants, reactivity for both laminin and HSPG was also localised within the explant in sites that were approximately similar in size and distribution to the pockets of ECM-like material seen by TEM. In FGF-treated explants, a few such regions were also detected; however, these were generally smaller and not as numerous as in explants treated with both growth factors. More sites exhibited reactivity for laminin than for HSPG and generally laminin reactivity was stronger.

In controls and TGF-treated explants no pockets of reactivity for laminin or HSPG were detected within the cellular layer. Thus the intercellular spaces revealed by TEM in TGF-treated explants did not contain ECM.

ß-crystallin accumulation. To assess fibre differentiation we measured the fibre-specific ß- and γ-crystallin content of explants at the end of the 5 day culture period by ELISA. Significant ß-crystallin accumulation occurred only in explants cultured with TGFß/FGF or FGF (P = 0.001, compared with control); an apparent enhancement of ß-crystallin accumulation in TGF/FGF-treated explants relative to the FGF-treated explants did not reach statistical significance. None of the treatments induced significant accumulation of γ-crystallin within the 5 day culture period.

Complementary immunolocalisation studies confirmed these findings and revealed that ß-crystallin appeared to be distributed throughout most cells in both TGFß/FGF- and FGF-treated explants.

Lens Explants from 21-day-old Rats: on Laminin Substratum

When explants were cultured cell surface down on a laminin substratum without growth factors, cells spread and migrated off the capsule onto the substratum forming an annulus around the explant. This process continued over the 5 day culture period and was significantly enhanced by FGF (Hales et al., 1992). The addition of TGFß, however, inhibited spreading and migration in the presence or absence of FGF so that a full annulus of cells did not develop; there were only a few isolated outgrowths of cells around the explant perimeter, and spreading and migration appeared to cease after 2 days of culture. This is consistent with the observation that the cells at the leading edge of these outgrowths had few of the pseudopodia characteristic of rapidly migrating cells seen in FGF-treated explants at 2 days. There was no apparent difference between TGFß- and TGFß/FGF-treated explants throughout the culture period.

During the first day of culture, all the cells in

TGFß-treated explants (that is, with or without FGF) had a morphology very similar to those in controls; however, by day 2 most of the cells that had spread onto the laminin substratum had become substantially elongated, some to the extent of being spindle-shaped or needle-like. In some regions cells that remained under the capsule also become elongated and aligned; these regions tended to extend between islands of epithelial-like cells. By 3 days of culture, explants treated with TGFß mostly consisted of elongated cells and under the capsule differences between the peripheral and central regions of the explants became detectable. The periphery was well populated with multilayers of aligned elongated cells, whereas cells in the central region were in reticular arrangements exposing regions of bare capsule.

Wrinkling of the capsule was noted in all explants cultured with TGFß under these explant conditions. The wrinkles had a reticular arrangement and were primarily located in the central region of the explant. The wrinkles were most obvious at 2 days and generally became less pronounced during the remainder of the 5 day culture period.

Cell loss also appeared to be a major feature of explants exposed to TGFß. Bare patches of capsule were initially detected in the central region of the explant at 3 days and condensed nuclei were readily visible in cells that had spread onto the laminin. Cell numbers then progressively decreased and by 5 days the majority of the cells had been lost from the explant; the remaining cells retained the reticular arrangement first observed at 3 days.

Lens Explants from Adult Rats: Standard Method

Phase contrast and SEM. The morphological changes observed by phase contrast microscopy in these experiments were essentially similar to those reported for the explants from 21-day-old rats cultured on laminin, although as expected under these culture conditions no cells migrated off the capsule. Throughout

the culture period there were no clear differences between TGFß- and TGFß/FGF-treated explants. During day 1, explants cultured with TGFß retained the cobblestone appearance characteristic of controls, but by 2 days many of the cells had elongated. Bare patches of capsule were detected at 3 days and these increased progressively during the culture period.

The latter finding was confirmed by SEM at 5 days which also revealed that the morphology of cells that remained in explants cultured with TGFß for 5 days was variable. Often cells were present in reticular arrays which seemed to consist mainly of mosaics of cells many of them epithelial-like. In other regions many cells were elongated and distinctly spindle- or needle-like and in some of these the cellular surface was covered with fine blebs. In the explant periphery, where more cells tended to survive, they were often present either as multilayers of smooth surfaced spindle-shaped cells or as more rounded cells with distinct surface blebbing typical of cells undergoing apoptotic cell death (Wyllie et al., 1980; Williams et al., 1992).

In FGF-treated explants, most cells retained an epithelial morphology although in the periphery some cells showed slight elongation characteristic of early fibre differentiation (Lovicu and McAvoy, 1992). Controls stayed as an epithelial monolayer throughout the culture period.

Immunohistochemical localisation studies. The pockets of laminin or HSPG reactivity reported above were not detected in explants from adult rats examined at the end of the culture period, irrespective of treatment. Reactivity for ß-crystallin was detected in some cells interspersed throughout the explant in controls and FGF-treated explants; in both TGFß and TGFß/FGF-treated explants the clumps of cells that survived for 5 days also included some cells that fluoresced for ß-crystallin. No γ-crystallin was detected in any of the explants. There was thus no evidence that any of the

treatments stimulated ECM production or fibre-specific crystallin accumulation during the 5 day culture period.

SUMMARY

TGFß induced cells in explants to undergo an extensive and rapid elongation which had features that distinguished it from FGF-induced fibre differentiation. TGFß also induced accumulation of extracellular matrix, capsule wrinkling, cell death by apoptosis and distinctive arrangements of cells. These TGFß-induced responses are characteristic of the changes reported to occur during formation of various types of cataracts (Novotny and Pau, 1984; Eshagian, 1982; Eshagian and Streeten, 1980; Green and McDonnell, 1985). Standard explants from 10-day-old rats responded to TGFß only in the presence of FGF. Comparable explants from adult rats, or from 21-day-old rats cultured on a laminin substratum, responded readily to TGFß whether or not FGF was present.

EXAMPLE 2

Detailed description of an explant study using an antibody against TGFß to inhibit TGFß-induced cataract-like changes.

METHOD

Lens epithelial explants (2 per culture dish) were prepared from 21-day-old rats and trimmed to remove the peripheral region as described elsewhere (See Example 1). Explants were preincubated in culture medium at 37°C in 5% CO_2/air for approximately 3 hours before use.

A pan-specific polyclonal antibody against TGFß (rabbit IgG; British Bio-technology, Abingdon, UK; Cat. No. BDA 47,) was used; this neutralises TGFß1, ß1.2, ß2, ß3, and ß5. This IgG and non-immune rabbit IgG were reconstituted in sterile phosphate-buffered saline to a concentration of 3 mg IgG/ml.

TGFß2 (Genzyme, Cambridge, MA) was diluted with sterile medium to a concentration of 0.25 ng/10 µl. Under sterile conditions, 33 µl immune or non-immune IgG solution was mixed with 20 µl TGFß2 stock solution and 47

μl medium, incubated at 37°C in 5% CO_2/air for 30 min, then diluted to 2 ml with medium. Preincubation medium was removed from two culture dishes and 1 ml TGFß-IgG mixture was added to each. All explants were cultured for 5 days with daily monitoring by phase contrast microscopy. Explants cultured with non-immune IgG served as controls for any effects of IgG itself on TGFß activity.

RESULTS

In the presence of non-immune IgG, TGFß induced rapid elongation which occurred within 2-3 days (Fig. 1a) and by 5 days cells had been lost from the explant revealing wrinkling of the underlying capsule (Fig. 1b). These changes are typical of changes described in detail in Example 1 for explants cultured with TGFß in the absence of IgG.

In the presence of anti-TGFß, these changes were completely blocked. Throughout the 5 day culture period the explants retained their original epithelial-like morphology (Fig. 1c, d) and were indistinguishable from explants cultured in medium alone.

References

1. Chamberlain CG, McAvoy JW. Induction of lens fibre differentiation by acidic and basic fibroblast growth factor (FGF). *Growth Factors*. 1989;1:125-134.

2. McAvoy JW, Chamberlain CG. Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development*. 1989;107:221-228.

3. McAvoy JW, Chamberlain CG, de Iongh RU, Richardson NA, Lovicu FJ. The role of fibroblast growth factor in eye lens development. *Ann NY Acad Sci*. 1991;638:256-274.

4. Hales A, Chamberlain CG, McAvoy JW. Measurement of lens epithelial cell migration on a laminin substratum using image analysis. *J Comput Assist Microscopy*. 1992;4:135-139.

5. McAvoy JW, Fernon VTP. Neural retinas promote cell division and fibre differentiation in lens epithelial explants. *Curr Eye Res.* 1984;3:827-834.

6. Parmigiani CM, McAvoy JW. The roles of laminin and fibronectin in the development of the lens capsule. *Curr Eye Res.* 1991;10:501-511.

7. Lovicu FJ, McAvoy JW. The age of rats affects the response of lens epithelial explants to fibroblast growth factor - an ultrastructural analysis. *Invest Ophthalmol Visual Sci.* 1992;33:2269-2278.

8. McAvoy JW. Cell lineage analysis of lens epithelial cells induced to differentiate into fibres. *Exp Eye Res.* 1988;47:869-883.

9. Lovicu FJ , McAvoy JW. Structural analysis of lens epithelial explants induced to differentiate into fibres by fibroblast growth factor (FGF). *Exp Eye Res.* 1989;49:479-494.

10. Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. *Internat Rev Cytol.* 1980;68:251-299.

11. Williams GT, Smith AS, McCarthy NJ, Grimes EA. Apoptosis: final control point in cell biology. *Trends Cell Biol.* 1992;2:263-267.

12. Mohan PS, Spiro RG. Macromolecular organization of basement membranes. Characterization and comparison of glomerular basement membrane and lens capsule components by immunochemical and lectin affinity procedures. *J Biol Chem.* 1986;261:4328-4336.

13. Novotny GEK, Pau H. Myofibroblast-like cells in human anterior capsular cataract. *Virchows Arch [Pathol Anat].* 1984;404:393-401.

14. Eshagian J. Human posterior subcapsular cataracts. *Trans Ophthal Soc UK.* 1982;102:364-368.

15. Eshagian J, Streeten B. Human posterior subcapsular cataract - an ultrastructural study of the posteriorly migrating cells. *Arch Ophthalmol.* 1980;98:134-143.

16. Green WR, McDonnell PJ. Opacification of the posterior capsule. *Trans Ophthalmol Soc UK*. 1985;104:727-739.

Variations and modification may be made in respect of the invention as above exemplified and defined in the following statement of claim:

1. A method of preventing or controlling cataract or cataract-like disorders in the eye of a mammalian subject which comprises administering to the subject an effective amount of one or more inhibitors of TGFß.

2. An ophthalmological formulation comprising one or more inhibitors of TGFß in a pharmaceutically acceptable carrier.

3. A method of preventing or controlling "aftercataract" formation in the eye of a mammalian subject following lens implant surgery which comprises implanting in the eye of the subject a lens coated with one or more TGFß inhibitors.

4. A lens implant coated with one or more TGFß inhibitors.

5. The use of inhibitors of TGFß in the manufacture of an ophthalmological formulation for preventing or controlling cataract or cataract-like disorders.

Dated this 19th day of November 1993

THE UNIVERSITY OF SYDNEY
By their Patent Attorneys
Griffith Hack & Co.

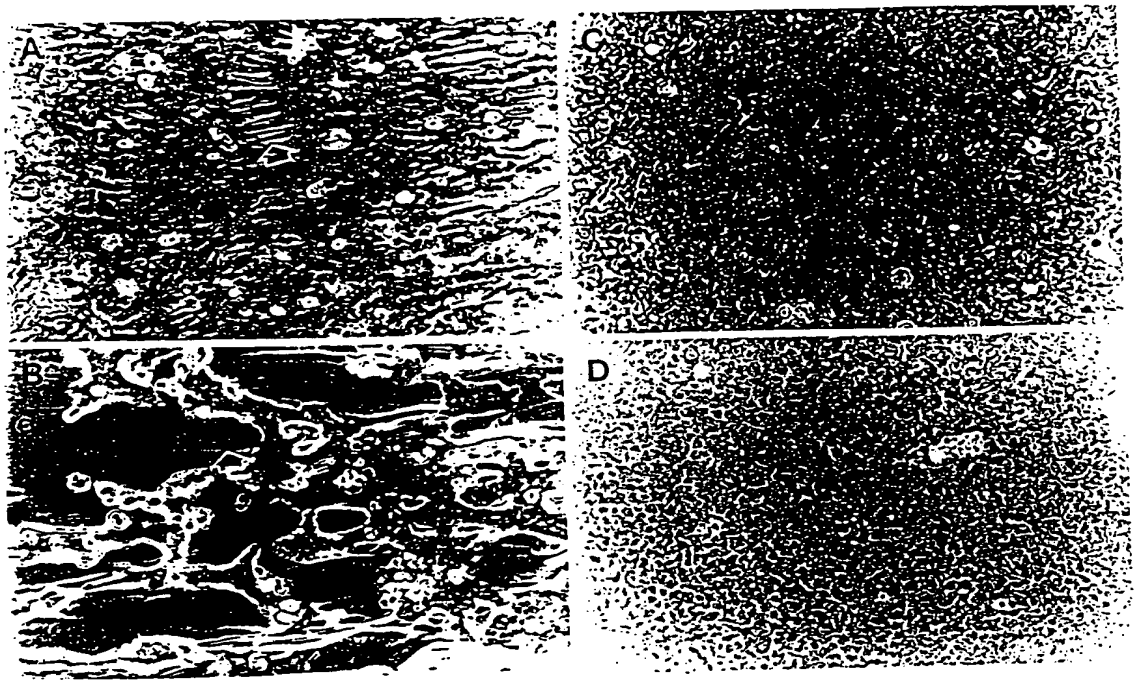

Figure 1. Phase contrast micrographs of lens epithelial explants from 21-day-old rats cultured with TGFß2 and non-immune IgG (a,b) or with TGFß and anti-TGFß IgG (c,d). Explants were photographed after 3 days (a,c) and 5 days (b,d) of culture. TGFß induces extensive elongation of cells (a, arrow); subsequently many cells are lost exposing regions of capsule which show wrinkles (b, arrow). Anti-TGFß completely blocks these changes and epithelial cells remain in a normal closely packed cobble-stone arrangement (c,d). The final concentrations of TGFß and IgG were 0.25 ng/ml and 50 µg/ml, respectively.